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THE ROLE OF CARBOHYDRATES IN MAMMALIAN SPERM-EGG INTERACTIONS: HOW IMPORTANT ARE CARBOHYDRATE EPITOPES?

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TABLE OF CONTENTS

1. ABSTRACT

Evidence implicating the involvement of carbohydrates in fertilization has been reported for decades in species which span the phylogenetic scale. The exact nature and role of these ligands in fertilization, however, has eluded investigators. Here, such investigations are reviewed as they relate to mammalian fertilization, with the principle focus on reviewing the role of carbohydrates involved in the primary binding event between sperm cell and egg.

2. INTRODUCTION

This manuscript reviews the role of carbohydrates in sperm-egg interactions in the mammalian fertilization process. Carbohydrates are thought to be prerequisite for sperm-egg binding to occur successfully. It has been noted in various species that sperm maturation involves the addition, subtraction, and/or alteration of sperm membrane glycoproteins as the sperm cell traverses the epididymus (1-5). Predictably so, changes in the lectin binding properties have been observed when comparing sperm from the testis, caput and caudal epididymus (6-9). However, a direct role of carbohydrates in the fertilization process has not been explicitly delineated. Upon deposition into the female reproductive tract, the sperm cell becomes capacitated as it approaches the site of fertilization. The sperm temporarily attaches to the isthmus mucosal surface and becomes sequestered within the mucosal folds, then is released from the isthmus and enters the ampulla to fertilize the egg (10-13). Plasma membrane changes are observed upon sperm capacitation, and include the removal and redistribution of peripheral and integral plasma membrane glycoproteins (14-18) resulting in the formation of plasma membrane domains (19-23).

The capacitated, acrosome-intact sperm cell which encounters an egg, passes through its cumulus oophorous, then reaches the zona pellucida, a glycoprotein matrix that coats the egg. Besides providing a microenvironment for the embryo, the zona pellucida also provides the primary binding site(s) for the spermatozoa to attach, and this binding triggers the acrosomal reaction (24-26). The zona pellucida of mice has been studied quite extensively on a molecular level and is comprised of three sulfated glycoproteins, namely ZP1, ZP2 and ZP3. ZP1 is primarily a structural protein, while ZP2 is involved in the secondary binding event. The roles of ZP1 and ZP2 will not be discussed here, rather, we will focus on the primary binding event mediated through ZP3. The reader is directed, however, to appropriate articles covering these former topics (27-30).

2. DISCUSSION

3.1. Ligand inhibition studies implicating the role of carbohydrates in sperm-zona pellucida binding

The primary sperm-egg binding event has been found to involve the ZP3 subunit of the mouse zona pellucida. The ability of different carbohydrates to inhibit sperm-egg binding has been studied in numerous species over the years and

Table 1. Inhibition of sperm-zona pellucida binding by well-defined carbohydrates

Species	Carbohydrate Residue	Inhibition, % Reference
Mouse	Sialic acid (M. musculus)	>95 at 100 mM[31]
	α-methyl mannose (M. caroli)	>95 at 100 mM
	Orosomucoid (M. musculus)	>95 at 20 mg/ml
	Orosomucoid (M. caroli)	<30 at 20 mg/ml
	Bi-antennary pentasaccharides terminating in α1,3-Gal	50 at 10 mM[32]
	Tri- and pentasaccharide derivatives of blood group B terminating in α1,3-Gal	50 at 1-5 mM
	Tetra-antennary terminating in α1,3-Gal	75 at 4 mM
	Galβ1,4GlcNAcβ1,4GlcNAc	~50 at 72 mM[33]
	Galα1,3Galβ1,4GlcNAc	>50 at 9 mM
	Both (combined)	~80
	Galα1,3Galβ1,4(Fucα1,3)GlcNAc	35 at 36 mM
	Galα1,3Galβ1,4(Fucα1,3)GlcNAc AND Galβ1,4GlcNAcβ1,4GlcNAc	60 at 36 + 72 mM, respectively
Rat	D-Mannose	~65 at 50 mM[36]
	α-Methyl mannose	>75 at 50 mM
	L-Fucose	50 at 80 mM
	α-Methyl mannose + L-fucose	50 at 14.3 mM
	Fucoidin	92 at 1 mg/ml
	Mannan	46 at 10 mg/ml
Hamster	N-Acetylglucosamine	100 at 250 mM – 1 mM
	N-acetylgalactosamine	100 at 250 mM – 1 mM
	N-Acetylmannosamine	100 at 250 mM – 1 mM
	Orosomucoid	0 at 4 mg/ml[37]
	Asialo-orosomucoid	100 at 2 mg/ml
	Agalacto-orosomucoid	100 at 4 mg/ml
	Fetuin	0 at 6 mg/ml
	Asialo-fetuin	100 at 3 mg/ml
	Ovo-mucoid	100 at 5 mg/ml
	Thyroglobulin	>95 at 5 mg/ml
	Submaxillary mucin	<5 at 8 mg/ml
	Asialo-submaxillary mucin	100 at 10 mg/ml
	Lactoferrin	>60 at 10 mg/ml
	Fucoidin	100 at 0.1 mg/ml
	Fucoidin fragments	85 at 0.1 mg/ml
Guinea Pig	Fucoidin	100 at 100 – 500 mg/ml[38]
	L-fucose	>70 at 50 mM
Human	Fucoidin	100 at 1 mg/ml[39]

these are synopsized in table 1. Homologous sperm-egg binding was found to be inhibited by different sugars as examined in two strains of mice (31). The most effective inhibition was noted in the presence of a millimolar concentration of sialic acid for *M. musculus*, while α-methyl mannose was most effective (also in millimolar concentrations) for *M. caroli*. Likewise, orosomucoid was a more effective inhibitor in *M. musculus* than in *M. caroli.* More recently, in a detailed comparison in *M. musculus* using unbranched and branched oligosaccharides of varying lengths and composition, Litscher et al (32) found that unbranched and biantennary oligosaccharides with terminal GlcNAc and galactose in a β-linkage were ineffective at inhibiting sperm-egg binding, whereas biantennary oligosaccharides with terminal α-galactose residues effectively inhibited binding in micromolar concentrations. Less effective were the tri- and pentasaccharide blood group B-related oligosaccharides, also ending in α-1,3-galactose, which showed inhibition in millimolar concentrations. Tetraantennary oligosaccharides terminating in either α- or β-galactose effectively inhibited binding in micromolar concentrations, whereas terminal β-GlcNAc-containing tetraantennary oligosaccharides of the same length were ineffective in inhibiting sperm-egg binding. Subsequently, a combination of tri- and

Table 2. Inhibition of sperm-zona pellucida binding by affecting carbohydrates of zona pellucida components

Species	Treatment	Inhibition,%	Ref.
Mouse	Galactosylation of zona pellucida by β1,4-galactosyl transferase	60	42
	Removal of terminal α-galactose residues using α-galactosidase	100	40
	O-linked oligosaccharides obtained by β-elimination	>80	40
	O-linked oligosaccharides oxidized using galactose oxidase	0	
	Subsequent reduction of O-linked oligosaccharides with $NaBH_4$	80	
	A comparison of the ability of various parts of ZP3 to inhibit sperm-egg binding		
	ZP3 intact, 50 – 100 nM	100	46-49
	AA $residues_{239-404}$, 120-200 nM	100	
	AA $residues_{239-404}$, lacking N-linked oligosaccharides and sialic acids, 120-200 nM	100	

tetrasaccharides were compared for their inhibitory ability. It was found that β-4- and α-3-galactosyl terminating structures inhibited sperm-egg binding in micromolar concentrations, and have been referred to as low and moderate affinity ligands, respectively (33). Fucosylation of these structures significantly increased the affinity of these ligands and results of mixing these inhibitors suggested the presence of low and high affinity oligosaccharide binding sites on sperm. Other studies have confirmed low and high affinity binding sites for sperm-egg binding in other studies in the mouse model (34-35).

Similar studies were performed in animals other than mice. In rats, α-methyl mannose was found to be the most effective monosaccharide inhibitor of sperm-egg binding, albeit at millimolar concentrations, while a synergistic effect was observed when fucose and α-methyl mannose were combined (36). Fucoidin, a polymer of sulfated fucose residues, was the most effective inhibitor found in this study. Studies performed in hamsters have shown that N-acetylated glucose, galactose and mannose residues successfully inhibit binding at high micromolar/low millimolar concentrations, while fucoidin and desialylated glycoproteins such as orosomucoid and fetuin proved to be effective inhibitors[37]. Furthermore, degalactosylated orosomucoid served as a viable inhibitor, supporting the participation of terminal N-acetylglucosamine residues in sperm egg binding. Consistent with these observations, appropriate glycosidase treatment of sperm (discussed later) also led to binding inhibition. Additionally, fucoidin was found an effective inhibitor of fertilization in guinea pigs and humans, suggesting a probable participation of fucose in mammalian gamete recognition (38-39).

While our understanding of sugar linkage and structural complexity requirements for sperm-egg binding has grown from these ligand inhibition studies, it appears that concise carbohydrate sequences are not critical for sperm binding. Leading carbohydrate recognition candidates on the mouse zona pellucida emerging from these and other studies include terminal galactose in an α- linkage (40), as well as β-N-acetylglucosamine. It was reported that galactosylation of ZP3 by the enzyme β-1,4 galactosyltransferase (in the presence of exogeneously added UDP-galactose) leads to an inhibition of sperm-egg binding *in vitro*. It is generally thought that the enzyme is present on the sperm surface and binds N-acetylglucosamine residues on ZP3, bringing sperm and egg together in a futile attempt to add galactose residues to ZP3 oligosaccharide termini (41,42). Mice generated in knockout studies which either lack the enzymes needed to generate or bind these termini, however, have no observable changes in fertility. That is, male mice lacking β1,4-galactosyl transferase and female mice lacking α1,3-galactosyl transferase have no notable changes in their fertilization capacity (43-45). These studies support the notion that redundant, overlapping interactions between sperm and egg contribute to species-related gamete recognition.

The inhibition of sperm-zona pellucida binding by specific carbohydrate ligands described above can exercise their effect by blocking carbohydrate epitopes present on ZP3 and/or complementary epitopes present on the sperm surface, as discussed below.

3.2. Carbohydrate epitopes of ZP3

A series of studies over the years have demonstrated that in murine ZP3 a small number of unique polypeptide sequences which are heterogeneously O-glycosylated are responsible for sperm-ZP3 binding. These data have been synopsized in table 2. Glycopeptides obtained from pronase digested murine ZP3 were found to retain sperm binding activity (46). In addition, removal of O-linked, but not N-linked, oligosaccharides led to a loss of sperm binding. The subsequent treatment of

Table 3. Inhibition of sperm-zona pellucida binding by affecting carbohydrates on sperm components

Species	Treatment	Inhibition, %	Ref.
Mouse	Pretreatment with substrate analogue of β1,4-galactosyltransferase, UDP-dialdehyde (2 mM)	80	64
Rat	Pretreatment of sperm with the α-mannosidase inhibitor, $Man_5GlcNAc$ (3.8 mM)	80	62-63
Hamster	α-Galactosidase	90	37
	α-L-Fucosidase	92	92
	β-N-Acetylhexosaminidase	85	85

these O-linked oligosaccharides with α-galactosidase or galactose oxidase destroyed sperm binding activity. The activity was restored upon subsequent reduction of the oligosaccharides with sodium borohydride, implicating the hydroxyl group of C_6 on the terminal galactose residues of these oligosaccharide chains in sperm binding activity (40-46). These O-linked oligosaccharide residues that are responsible for sperm binding are located on a cluster of serine residues on the carboxy terminus of the ZP3 molecule (47-49). ZP3 glycopeptides were found to bind more strongly than free O-linked oligosaccharide chains, suggesting the advantage of steric positioning of critical carbohydrate residues as they are presented within the context of a protein. Removal of sialic acid residues does not affect sperm binding activity (49).

Biochemical purification and cloning endeavors from a multitude of laboratories have led to the identification and characterization of ZP3 homologs in a variety of species (50-58). A striking overlap of predicted secondary structures is observed when comparing hamster and mouse ZP3 amino acid sequences (59). Other interspecies comparisons suggest conservation of disulfide and potential N-linked glycosylation sites. Variations in glycosylation patterns are better tolerated for the retention of biological activity in some species than others. When hamster ZP3 is expressed in mouse teratocarcinoma cells, for instance, it lacks biological activity, presumably due to inappropriate glycosylation, whereas expression of mouse ZP3 in a variety of non-oocyte cell lines produces a biologically active molecule, in spite of different glycosylation patterns (60). Despite this former observation, when hamster ZP3 is expressed in transgenic mice, functional hybrid macromolecules are synthesized, consisting of mouse and hamster ZP3 subunits, both of which retain sperm binding activity (61). It can be speculated that effective sperm-zona pellucida binding involves multivalency, resulting from a combination of multiple interactions with varying affinities, some of which may be protein-carbohydrate, carbohydrate-carbohydrate, or protein-protein interactions.

3.3. Carbohydrate epitopes of zona pellucida binding proteins on sperm

Many oocyte binding proteins have been identified in sperm, some of which are glycosylated and have lectin binding domains. Table 3 lists the effects of sperm carbohydrate manipulations on sperm-zona pellucida binding. Glycosidase treatment of sperm has been found to diminish their ability to bind to the zona of various species. The inhibitory effect of these treatments may be due to a generic perturbation of the sperm surface, or perhaps by directly affecting zona pellucida binding ligands on the sperm surface. The enzyme α-D-mannosidase has been found on the rat, mouse, hamster and human sperm, with its active site in the proper orientation so that it is believed to function directly in sperm-zona pellucida binding (62-63). The sperm surface enzyme β-1,4 galactosyltransferase, which has already been described above, is believed to directly bind its substrate on the zona pellucida and thereby participate in sperm-zona pellucida binding (64).

Zona pellucida binding candidate proteins present on the sperm surface are listed in table 4. Here a brief description of leading zona pellucida binding candidates is provided from various species. SP-56 is a peripheral plasma membrane protein located on the head of mouse sperm and has been affinity purified using mouse ZP3 (65-66). Photoaffinity crosslinking studies have demonstrated that SP-56 binds O-linked glycopeptides of ZP3. A 95 kd protein has been identified which is present in capacitated and non-capacitated sperm, is tyrosine phosphorylated during capacitation, and binds ZP3 (67). The degree of tyrosine phosphorylation was found to be dependent on the zona pellucida concentration. In the guinea pig, a sperm specific plasma membrane glycoprotein, designated Sperad, was identified by screening a cDNA expression library with anti-sperm plasma membrane antiserum (68). This protein resembles a family of liver cell adhesion molecules and is thought to be involved in

Table 4. Sperm glycoprotein candidates involved in zona pellucida binding

Species	Component	Description[Reference]
Mouse	SP-56	Sperm surface protein purified by ZP3 affinity chromatography. Photoaffinity crosslinked to ZP3 O-linked oligosaccharides [65,66]
	β1,4-Galactosyltransferase	Blocking or inhibiting enzyme activity diminishes sperm-egg binding[41,42,64]
	95 Kd sperm protein	This protein undergoes tyrosine phosphorylation in a ZP concentration dependent manner[67]
Rat	α-D-Mannosidase	Inhibiting enzyme activity blocks sperm-egg binding
Guinea Pig	Sperad	Integral membrane glycoprotein presumably related to biliary putative cell adhesion molecules[68]
	PH-20	Sperm surface glycoprotein which is present on the inner acrosomal membrane and sperm surface of posterior spermhead, involved in ZP adhesion. Also exhibits hyaluronidase activity. A wide distribution of this gene has been reported among mammals [69-71].
	Zona and fucoidan binding proteins	These are thought to be proacrosin/acrosin molecules which are retained on the acrosomal membrane of sperm[72].
Porcine	Proacrosin	Broad specificity carbohydrate binding protein, requires clustering of saccharide residues[73,74].
	Fucose binding protein	Localized to apical region of sperm head[75].
	Apz	An integral plasma membrane protein was purified using wheat germ agglutinin lectin chromatography. Monovalent α-Apz completely blocks sperm-egg binding[76].
	Porcine spermadhesins (AQN and AWN family)	Mosaic, peripheral sperm membrane progeins, containing zona pellucida-, serine proteinase-inhibitor- and heparin-binding sites[77-80].
	SP-38	Competes with proacrosin for ZP binding, both of which are inhibited by fucoidan[81].
	Zonadhesin	Zona binding protein containing mucin-like domains and prepro von Willebrand factor domains[82].
Rabbit	Rabbit sperm antigens	Family of intrinsic membrane glycoproteins with lectin-like zona binding activity, whose members share amino acid and carbohydrate similarities. Residues include xylose and sialic acid, with glucosamine as the only detectable amino sugar[83-85].
	Testis hepatocyte lectin 2/3	Galactosyl receptor, immunologically related to the rat liver asialoglycoprotein receptor, also has rabbit zona binding activity[86].
Human	FA-1	Purified antigen blocks sperm-egg binding in humans. Periodate Schiff positive. Contains 19% carbohydrate residues. Binds Lens culinaris and Concanavalin A. also contains phosphorylatable tyrosines. Recombinant FA-1 antigen devoid of carbohydrates binds native, glycosylated ZP3 in mouse[87-90].

heterotypic cell interactions between sperm and egg. A sperm surface antigen, PH-20, has been identified in the guinea pig which is a sperm surface protein that migrates from the posterior head surface to the inner acrosomal membrane following acrosome exocytosis, then undergoes endoproteolytic cleavage and exhibits hyaluronidase activity (69-70). A monoclonal antibody against this antigen inhibits sperm-egg binding, and it is thought that PH-20 is involved directly in sperm-egg binding (71). Other putative zona pellucida receptors have been identified in the guinea pig by their ability to bind radioactively labeled zona proteins as well as fucoidan (72). These proteins range from 95 to 18 kDa in molecular weight and some have been identified as proacrosin/acrosin.

Many zona pellucida binding proteins have been identified in the pig. A broad specificity carbohydrate binding protein has been identified as proacrosin in boar sperm, which has been proposed to consolidate binding of sperm to the egg (73-74). A fucose binding protein has been localized to the apical region of the sperm head (75). An integral sperm membrane protein, Apz, which is distinct from proacrosin, has lectin binding properties and is thought to be associated with the cytoskeleton of sperm (76). Anti-Apz antibodies block sperm-egg binding. A family of low molecular weight, sperm associated carbohydrate and zona binding proteins, the spermadhesins, have also been characterized. Members of this family include AWN-1, a broad

Table 5. Evidence for sperm-zona binding independent of carbohydrates

Species	Component	Description[Reference]
Mouse	SP-56	Has no carbohydrate recognition domain, but has Sushi and C4b-binding domains [65,66].
	Monoclonal antibodies IE1, IE2, IE3, IE4, IE6	These antibodies block fertilization in the mouse, but recognize epitopes on ZP2 and/or ZP3 that do not contain galactose[91-93].
	Proteinase inhibitor binding protein	>80% inhibition of sperm egg binding occurred in the soybean trypsin inhibitor sites (8 mg/ml)[77-80].
Porcine	Zonadhesin	Testis specific mosaic protein, mucin domain removed prior to sperm surface expression; Demonstrated species specific binding to porcine zona pellucida[82].

specificity sperm surface lectin, which also contains soybean trypsin inhibitor and heparin binding sites. Interestingly, N- or O-glycosylation of this molecule diminishes its ability to bind zona pellucida (77-80). A calcium dependent zona binding sperm membrane protein, SP-38, was found to compete with proacrosin for early egg binding in pigs (81). Zonadhesin, an integral sperm membrane protein, was found to bind zona pellucida in a species-specific manner in the pig (82). This protein contains mucin-like as well as prepro von Willebrand factor domains.

In the rabbit, a lectin-like, high affinity zona binding protein family, rabbit sperm autoantigen (RSA), has been identified which binds sulfated, complex carbohydrates including fucoidin, dextran sulfate, chondroitin sulfate B and heparin. RSA, as well as antibodies against RSA, inhibit sperm-egg binding *in vitro* (83-85). Another carbohydrate binding protein, the rabbit testis galactosyl receptor, is related to the liver asialoglycoprotein receptor, and binds galactosyl residues as well as rabbit zona pellucida proteins (86). This protein has also been identified in the rat testis. In humans, a fertilization antigen, FA-1, has been identified using a monoclonal antibody which was generated against the human germ cell plasma membrane (87). The affinity purified antigen, the monoclonal antibody and its Fab' fragments were found to block sperm-zona pellucida binding in humans (88). The cDNA encoding the FA-1 antigen has been cloned from the mouse testis, sequenced, and determined to be testis specific in its expression (89). This antigen is being examined for its potential in developing a contraceptive vaccine for humans (90).

3. PERSPECTIVE

While the majority of candidate zona binding proteins from sperm apparently interact with the zona pellucida through carbohydrate interactions, suggested by inhibition studies and by the identification of lectin binding domains on the sperm proteins, a number of sperm proteins contain non-carbohydrate related functional domains as well. This suggests the possibility of the involvement of some binding events between the sperm cell and egg that are independent of carbohydrates. Sperm antigen SP-56, although found to be photoaffinity crosslinked to the O-linked oligosaccharide regions of ZP3, contains no obvious carbohydrate binding domain, but instead has Sushi and C4b binding domains. It has been proposed that, on the porcine sperm, Zonadhesin's mucin-like domain is removed prior to egg binding. Additionally, it was found that blocking the protease inhibitor binding site of the sperm protein Spermadhesin inhibits its zona binding activity, although the two binding sites appear to overlap sterically. These carbohydrate independent binding sites are listed in table 5 and may suggest that zona pellucida binding events involving carbohydrate interactions may also involve high affinity protein-protein interactions. Recombinant proteins which are glycosylated may serve as better antigens than non-glycosylated proteins for contraceptive vaccine development (94-96). This may be due to the ability of glycosylated proteins to attain a more biologically appropriate conformation for antigen presentation as well as having an increased biological halflife. With the ability of recombinant proteins which lack carbohydrates to undergo successful binding to zona components (89, 97) *in vitro*, as well as the ability of relevant transgenic/gene-ablated mice to undergo fertilization *in vivo* (43-45), it is becoming apparent that, while carbohydrates may be involved in sperm-egg interactions, their absolute and essential role in primary sperm-egg binding still remains in question.

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